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Assessing protection against OP pesticides and nerve agents provided by wild-type HuPON1 purified from *Trichoplusia ni* larvae or induced via adenoviral infection

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ABSTRACT

Human paraoxonase-1 (HuPON1) has been proposed as a catalytic bioscavenger of organophosphorus (OP) pesticides and nerve agents. We assessed the potential of this enzyme to protect against OP poisoning using two different paradigms. First, recombinant HuPON1 purified from cabbage loopers (iPON1; *Trichoplusia ni*) was administered to guinea pigs, followed by exposure to at least 2 times the median lethal dose (LD_{50}) of the OP nerve agents tabun (GA), sarin (GB), soman (GD), and cyclosarin (GF), or chlorpyrifos oxon, the toxic metabolite of the OP pesticide chlorpyrifos. In the second model, mice were infected with an adenovirus that induced expression of HuPON1 and then exposed to sequential doses of GD, VX, or (as reported previously) diazoxon, the toxic metabolite of the OP pesticide diazinon. In both animal models, the exogenously added HuPON1 protected animals against otherwise lethal doses of the OP pesticides but not against the nerve agents. Together, the results support prior modeling and *in vitro* activity data which suggest that wild-type HuPON1 does not have sufficient catalytic activity to provide *in vivo* protection against nerve agents.

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1. Introduction

Wild-type human paraoxonase-1 (HuPON1) is a six-fold beta-propeller protein containing two calcium ions [1]. This protein is produced in the liver and is found in the plasma in association with high density lipoprotein (HDL) particles [2]. HuPON1 is a promiscuous enzyme with activity against several classes of substrates including arylesters, lactones, and OP compounds such as pesticides and nerve agents [3–7]. The catalytic activity of wild-type HuPON1 against particular OP compounds is influenced by the naturally occurring allelic variants of arginine or glutamine at amino acid residue 192 [8,9]. Likewise, a variety of other amino acid substitutions in and around the putative active site have been shown to alter both the catalytic efficiency and the fine specificity of HuPON1 for OP substrates [6,10–13]. PON1 is believed to play an important role in determining the resistance or susceptibility to OP pesticide poisoning, in that PON1 knockout mice are dramatically more susceptible to the toxic metabolites of the OP pesticides diazinon and chlorpyrifos (diazoxon and chlorpyrifos oxon, respectively; Fig. 1) than control mice [14]. Likewise, mice infected with an adenovirus that induces endogenous expression of HuPON1 were found to be more resistant to diazoxon [15]. Because currently approved therapeutic drugs for treatment of OP nerve agent exposure do not directly interact with and reduce the concentration of nerve agent in the bloodstream, there is great interest in identifying protein-based "bioscavenger" drugs that can catalytically detoxify and degrade OP nerve agents in the blood [16]. However, the extent to which wild-type HuPON1 is able to afford protection against OP nerve agents *in vivo* remains unclear. Several groups have suggested that the catalytic efficiency and stereoselectivity of HuPON1 for G- and V-type nerve agent are inadequate to protect against poisoning by these compounds [17,18]. In contrast, injection of exogenous wild-type HuPON1 has recently been reported to protect rats against poisoning by a microinstillation exposure to $1.2 \times LD_{50}$ of the nerve agents GB or GD [19–22]. In the current study, two approaches were used to evaluate the protective efficacy of HuPON1 against OP nerve agent poisoning. First, guinea pigs were injected with purified HuPON1 from *Trichoplusia ni* larvae (iPON1) and exposed to GA, GB, GD, or GF (Fig. 1). Second, mice were infected with an adenovirus that induces HuPON1 expression and subsequently exposed to GD or VX.

Abbreviations: OP, organophosphorus; HuPON1, human paraoxonase 1; i-PON1, insect larvae-produced recombinant human PON1.

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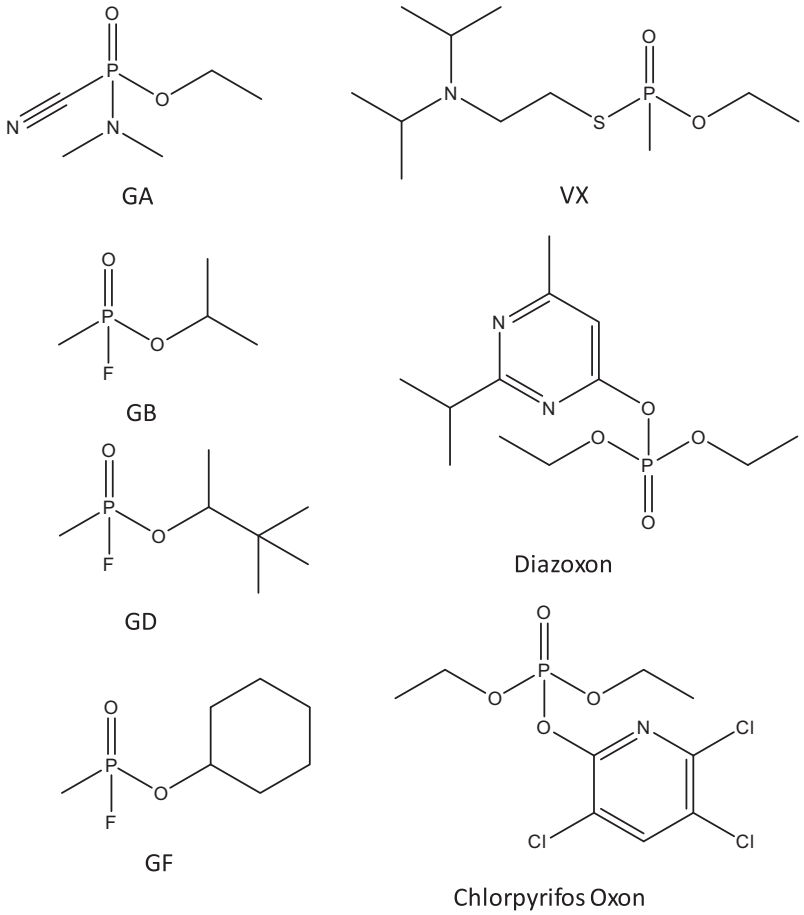

Fig. 1. Structures of OP compounds. The seven OP compounds utilized in this study are shown.

2. Materials and methods

2.1. Reagents

Insect-cell-produced iPON1 (with methionine at amino acid 55 and glutamine at amino acid 192) was purchased in purified form (>95% pure based on protein gel analysis [23]) from Chesapeake Protein Expression and Recovery Laboratories (C-PERL, Savage, MD). Adenoviral vectors encoding HuPON1 (Ad-PON1, encoding L55 and Q192), human butyrylcholinesterase (Ad-BuChE; used as a positive control) or an empty vector (as a negative control) were generated as previously described [15]. OP nerve agents GA (ethyl N,N-dimethylphosphoramidocyanidate), GB (2-(fluoro-methylphosphoryl)oxypropane), GD (3,3-dimethylbutan-2-yl methylphosphonofluoridate), GF (cyclohexyl methylphoshonofluoridate), and VX (*O*-ethyl-S(2-diisopropylaminoethyl) methylphosphonothiolate) were obtained from the US Army Edgewood Chemical Biological Center (ECBC, Aberdeen Proving Ground, MD) at greater than 98% purity and were diluted into saline prior to use. Chlorpyrifos oxon and diazoxon were purchased from Chem Services (West Chester, PA).

2.2. Guinea pig efficacy experiments

Male Hartley guinea pigs (300–400 g, from Charles River Laboratories, Wilmington, MA) were provided with carotid catheters. After acclimating to the animal facility, animals were injected via catheter with either 5 or 10 mg/kg of iPON1 diluted in saline. For pharmacokinetic analyses, blood samples were collected via toenail clip and immediately processed to separate plasma. Samples were collected from three animals administered 10 mg/kg iPON1 at 5, 15, 30, 60, 120, 240, and 1440 min after enzyme injection. Plasma samples from each animal were also collected prior to iPON1 injection to serve as background controls. The presence of iPON1 in guinea pig plasma samples was detected by tracking the production of *p*-nitrophenol (at A_{412}) from paraoxon (9 mM in 50 mM Tris, 10 mM $CaCl_2$, pH 7.4), as previously described [24]. To correlate the activity in each sample to a concentration, these results were compared to a standard curve generated using purified iPON1 (0.8–24.5 μg of protein) under the same assay conditions. For efficacy experiments, guinea pigs ($n = 2$ for each nerve agent; $n = 4$ for chlorpyrifos) were injected with 5 mg/kg of iPON1, followed 5 min later by exposure via subcutaneous injection to $2 \times LD_{50}$ of GA, GB, GD, or GF (240, 87, 56, or 108 μg/kg, respectively) or to $\sim 4 \times LD_{50}$ of chlorpyrifos oxon (250 mg/kg). Animals were monitored for signs of OP poisoning, and survival was scored 24 h after OP exposure.

2.3. Mouse efficacy experiments

Male Swiss Webster mice (20–25 g) were purchased from Charles River Laboratories. Amplified Ad-PON1 or Ad-BuChE was injected into the tail vein of mice (2.0×10^{11} viral particles/mouse), as previously described [15]. Starting at day 4 post-infection (when induced protein expression was at maximal levels), the mice were exposed to $2 \times LD_{50}$s of GD (220 μg/kg), VX (24 μg/kg), or (as previously reported) diazoxon via subcutaneous injection. The data presented show the number of animals that survived 24 h after OP exposure.

3. Results and discussion

3.1. Pharmacokinetics of iPON1 in guinea pigs

Guinea pigs were injected via carotid catheter with 10 mg/kg of iPON1, and the circulatory stability was determined by collecting blood samples over a 24-h period and analyzing them for paraoxonase activity. The results shown in Fig. 2 reveal a maximum iPON1 concentration of approximately 300 ng/μL in plasma at 5 min post-injection. This value closely approximates the maximal concentration of iPON1 (~275 ng/μL) that would be predicted if 100% of the injected enzyme was bioavailable. The enzyme cleared rapidly from circulation, with a half-life of roughly 1 h; this rapid clearance (relative to endogenous PON1) was presumably due to differences in post-translational modifications between iPON1 and endogenously produced enzyme [23]. None of the injected animals displayed any abnormal behavioral signs or other consequences of enzyme administration for at least 7 d after injection, suggesting that iPON1 is safe to administer to guinea pigs in the dose range used.

3.2. Protection by iPON1 injection against OPs

Guinea pigs were injected as described above with iPON1. At 5 min after enzyme injection (when the majority of the injected iPON1 was bioavailable in the plasma), animals were exposed via subcutaneous injection to $2 \times LD_{50}$ of the nerve agent GA, GB, GD, or GF. In parallel, guinea pigs were exposed to $\sim 4 \times LD_{50}$ of chlorpyrifos oxon. As shown in Table 1, 75% of the animals exposed to chlorpyrifos oxon survived to 24 h. These surviving animals displayed minor and transient signs of OP poisoning (salivation and convulsive chewing) that resolved within 1 h of OP exposure, while the remaining animal died between 8 and 16 h after chlorpyrifos oxon exposure. In data not shown, animals injected with lower doses of iPON1 displayed delayed onset of signs of OP poisoning, and animals that died had a substantially delayed time-to-death as compared to untreated controls; untreated guinea pigs exposed to the same dose of chlorpyrifos oxon survived no more than 15 min post-exposure.

In contrast to results obtained with chlorpyrifos oxon, all of the guinea pigs injected with iPON1 and then exposed to $2 \times LD_{50}$ of GA, GB, GD, or GF displayed severe signs of OP poisoning (convulsions, labored breathing) within minutes of OP exposure. All of these animals died in the same time frame as had previously been observed for untreated animals, suggesting that the administered iPON1, while able to protect against chlorpyrifos oxon, did not afford detectable protection against the OP nerve agents.

Table 1
24-Hour survival rates of OP-exposed guinea pigs injected with iPON1.

	GA	GB	GD	GF	Chlorpyrifos oxon
iPON1	0/2	0/2	0/2	0/2	3/4[a]

[a] The animal that died had a delayed time to death relative to untreated guinea pigs.

Table 2
24-Hour survival rates of OP-exposed mice 4 d after adenoviral infection.

	GD	VX	Diazoxon[b]
Control	0/5	0/6	0/3
Ad-PON1	0/8	0/6	6/6
Ad-BuChE	ND[a]	4/4	ND[a]

[a] ND = not determined.
[b] Reproduced from reference [15].

3.3. Protection by Ad-PON1 infection against OPs

We have previously described the use of adenovirus in mice to induce expression of HuPON1 [12] or human BuChE [21] and have demonstrated that these virally induced proteins can afford protection against OP pesticides and nerve agents [12,22], respectively. As shown in Table 2, none of the mice infected with a negative control (empty vector) adenovirus 4 d prior survived exposure to $2 \times LD_{50}$ of GD, VX, or diazoxon. When pre-treated with Ad-HuPON1, which resulted in elevation of plasma PON1 levels by 10–30-fold over control pretreated mice, six out of six animals survived exposure to $2 \times LD_{50}$ of diazoxon (12), but none survived exposure to $2 \times LD_{50}$ of either GD ($n = 8$) or VX ($n = 6$). As a positive control, mice ($n = 4$) were infected with Ad-BuChE and exposed to $5 \times LD_{50}$ of VX. All of these control animals survived with no detectable signs of OP poisoning. Together, these data indicate that induction of HuPON1 expression by adenoviral infection can provide protection to mice against diazoxon [12] but not GD or VX.

4. Conclusions

Although wild-type HuPON1 was able to protect animals from multiple lethal doses of two different OP pesticides, the enzyme failed to provide protection against $2 \times LD_{50}$ of the nerve agents GA, GB, GD, GF, or VX. These results are particularly striking in light of the similar molecular weights (140–350 daltons) and dramati-

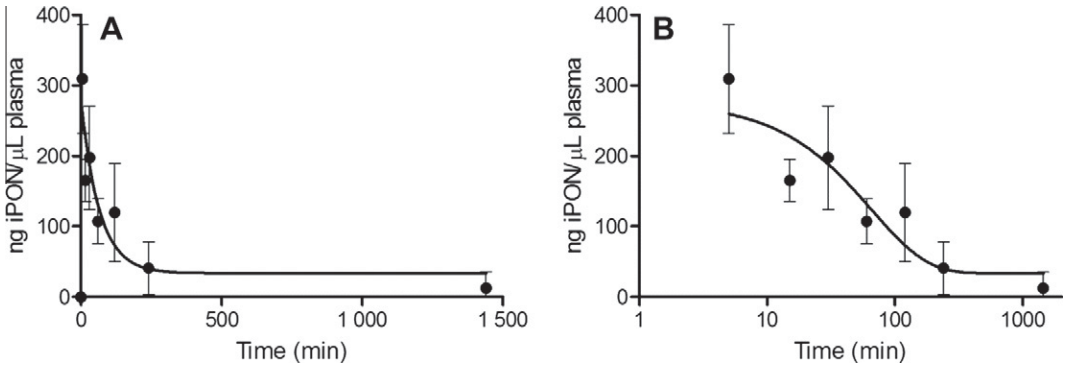

Fig. 2. Pharmacokinetic profile of iPON1 in guinea pigs. The amount of iPON1 present in the plasma of guinea pigs ($n = 3$) injected via carotid catheter with 10 mg/kg of iPON1 is shown. Panel A depicts the stability of injected iPON1 on a linear timescale over the course of a 24-h period. Panel B displays the same data on a logarithmic timescale, to show that appreciable amounts of enzyme are present in plasma for at least 90 min after injection. The half-life ($T_{1/2}$) is approximately 60 min. Data are shown after subtraction of endogenous PON1 activity level from each animal. Results show average ± standard deviation (SD) for each value.

cally different LD_{50} doses of the pesticides relative to the nerve agents. For example, the dose of chlorpyrifos oxon survived by guinea pigs injected with iPON1 was ~730 μmoles/kg, while the dose of GD that resulted in death for all animals was only ~300 nmoles/kg, or more than 2000-fold fewer OP molecules. The results presented here support previous modeling predictions indicating that wild-type human PON1 may have sufficient catalytic activity to afford *in vivo* protection against some OP pesticides, but is not able to protect against even relatively modest ($2 \times LD_{50}$) doses of OP nerve agents. Consistent with this conclusion is the recent observation that HuPON1 knockout mice (which are more susceptible to diazoxon and chlorpyrifos oxon toxicity than are wild-type mice [11]) are not more susceptible to nerve agent poisoning. Additionally, HuPON1-expressing transgenic mice are not more resistant to OP nerve agents (Cerasoli et al., unpublished data). Despite the failure of wild-type HuPON1 to provide protection against OP nerve agent toxicity, this enzyme does show great promise as a structural scaffold on which to design novel variants with enhanced activity against a broad spectrum of OP compounds.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), and the Animal Welfare Act of 1966 (P.L. 89-544), as amended.

The views expressed in this manuscript are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government.

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